Site-directed mutagenesis and expression in *Escherichia coli* of WMAI-1, a wheat monomeric inhibitor of insect α-amylase

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Abstract

The wheat monomeric inhibitor WMAI-1 (syn. 0.28) produced in *Escherichia coli* using the pT7-7 expression vector has the correct N-terminal sequence and the same electrophoretic mobility and specific activity towards the α-amylase from the insect *Tenebrio molitor* as the native WMAI-1 isolated from wheat. This confirms that the native inhibitor is not glycosylated and contradicts claims that a putative glycosyl moiety was essential for inhibition. Thirteen mutants have been obtained at six different sites. Substitution of the highly conserved N-terminal S by the sequence ARIRAR increased the pre-incubation time required for maximum activity. A similar result was obtained by insertion of GPRLPW after position 4, while insertion of EPRAPW at the same position rendered the inhibitor inactive. The substitution D/EGPRL and insertions DGP or D, at position 58, produced complete inactivation. All other mutations had only minor effects on activity.

Introduction

A single protein family from cereals includes trypsin inhibitors and monomeric, dimeric, and tetrameric inhibitors of heterologous α-amylases [4, 5]. These inhibitors, which are quite abundant in endosperm, are of interest because of their possible role as plant defence proteins [4, 5, 7, 10, 16, 18] and because they are major allergens involved in baker's asthma disease [1, 8, 22]. Although there is considerable information concerning the reactive sites and other relevant features of protease inhibitors (see [4]) little is known about the key structural details responsible for α-amylase inhibition.

The wheat monomeric α-amylase inhibitor WMAI-1 (syn. 0.28) is among the best characterized members of this family. It is highly active against the α-amylase of the insect *Tenebrio molitor* and has little activity against the human salivary α-amylase [4, 15]. Its amino acid sequence has been determined by direct protein sequencing [12, 22] and the existence of five disulphide bridges has been inferred [15, 22]. It has been claimed that it is a glycoprotein with one reducing sugar per molecule [15, 18] and that this sugar plays an important role in the inhibition mechanism, which involves the interaction of two molecules of inhibitor with one molecule of enzyme [19]. Failure to confirm the glycoprotein

nature of this inhibitor by standard biochemical methods (G. Salcedo, R. Sanchez-Monge and L. Gomez, unpublished) has led to the experiments reported here, which involve the production of active inhibitor by expression of its cDNA in *Escherichia coli* and the identification of parts of the molecule that are critical for the inhibition by site-directed mutagenesis.

Materials and methods

Construction of a WMAI-1 expression plasmid

A cDNA encoding WMAI-1 cloned in the *Sma* I site of plasmid pUC12 (clone pUP28) was provided by C. Maraña. Expression vector pT7-7 [21] was the gift of S. Tabor (Boston, MA). An expression plasmid was constructed by subcloning a *Sty* I/*Bam* HI fragment from plasmid pUP28 together with a synthetic *Nde* I/*Sty* I adaptor oligonucleotide in the *Nde* I/*Bam*HI sites of plasmid pT7-7 (Fig. 1A).

Mutagenesis of WMAI-1

Mutant A1 was generated by subcloning of a *Bst* XI/*Pst* I fragment from plasmid pUP28, made blunt at the *Bst* XI end, into the *Sma* I/*Pst* I sites of the pT7-7 polylinker (Figs. 1A and 2A).

Mutants C were obtained by oligonucleotide-directed mutagenesis (Amersham kit RPN1523), using 43-mer synthetic oligonucleotide I:

TCGGGCACCTGACTCAGA$\overset{C}{\underset{A}{G}}$GGGGGCCC-GATGCCCACAGACTGGA.

Mutants B, D, E, and F were constructed using the WMAI-1 expression plasmid as parental material. Following cleavage with the indicated enzyme (Fig. 2A), the linear plasmids were made blunt by filling with Klenow (mutants B, D, and E) or by recessing with T4 polymerase (mutants F), and ligated in the presence of the corresponding double-stranded linker molecule: II, GGGCCCGAGGCTCC (both orientations; mutants B1, B2, D1 and D2); III, GGGCCCGAGGCTC (in mutants E and F). Linker III introduced a frame shift, so the in-frame mutants recovered were spontaneous rearrangements in which the reading frame had been restored. A first screening of the recombinants (except A1 and E3) was made by checking the creation of an *Apa* I site. Putative recombinants were further checked for the expression of the recombinant proteins and then by sequencing. The Sanger method [16] was used either after subcloning in M13 or directly in the expression plasmid, using a primer for the T7 RNA polymerase promoter (Boehringer). In two cases, E2 and F1, incorporation of partial linkers occurred. In mutant E1, the correct reading frame was maintained because of the deletion of C, likely due to residual 3′–5′ exonuclease activity in the Klenow fragment preparation.

Expression and purification of proteins

Transformation with the different plasmids was carried out in *E. coli* HMS174 (gift of T. Ruiz-Argüeso, Madrid) to avoid instability problems. *E. coli* BL21 (DE3), which contains a lac-inducible RNA-polymerase gene, was used for protein expression.

Cultures grown at 37 °C to $A_{660} \approx 0.4$–0.5, induced with isopentenylthiogalactoside (IPTG) for 2–4 h, centrifuged, resuspended in buffer (10 μM Tris-HCl, pH 7.5, 20% w/v sucrose, 15 mM EDTA) and incubated on ice for 30 min. Cells were harvested, resuspended in distilled water and sonicated. A cytosolic fraction and a pellet containing inclusion bodies were obtained by centrifugation at 15000 × *g* for 20 min. Inclusion bodies were washed with buffer A (50 mM Tris-HCl pH 7.5, 5% glycerol) containing 2 M NaCl and extracted with 2 M guanidinium chloride in the same buffer. After dialysis against buffer A with 1 M NaCl, the extracted proteins were precipitated with 70% $(NH_4)_2SO_4$ and resuspended in 0.1 M acetic acid, dialysed and freeze-dried. Final purification was achieved by reversed-phase high-performance liquid chromatography (RP-HPLC) on an Ultrapore RPSC C3 column (particle size 5 μm; 1 cm × 25 cm; Beckman) using a

20–30% linear gradient of acetonitrile in H_2O containing 0.1% trifluoroacetic acid.

Enzyme inhibition tests

Inhibitory activity against the α-amylase from larvae of *Tenebrio molitor* was tested using a buffer containing 20 mM sodium acetate pH 5.4, 100 mM NaCl, 0.1 mM $CaCl_2$ as described by Gutierrez *et al.* [10]. The α-amylase and the inhibitor were preincubated at 25 °C for the indicated times. Previously reported methods were used to assay trypsin [2, 3] and chymotrypsin [11].

Other methods

Protein concentrations were determined by the method of Smith *et al.* [20]. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemli [13]. N-terminal amino acid sequences were performed by automated Edman degradation by C. Buesa (University of Barcelona).

Results

Production of WMAI-1 in Escherichia coli

The sequence of the insert in plasmid pU28 (C. Maraña *et al.*, unpublished) encoded a protein with the same amino acid sequence reported for WMAI-1 [12, 22], except for a GD deletion after position 114. A *Sty* I-*Bam* HI fragment of the WMAI-1 cDNA clone was subcloned in the pT7-7 plasmid, between the *Nde* I and *Bam* HI sites, using an *Nde* I-*Sty* I adaptor oligonucleotide which reconstructed the cDNA sequence coding for the N-terminal of the WMAI-1 mature protein, plus an N-terminal methionine, thus eliminating the signal peptide (Fig. 1A). Cultures of *E. coli* (BL21) transformed with the expression plasmid and induced with IPTG showed an extra band when analysed by SDS-PAGE that was absent in induced cultures containing the plasmid

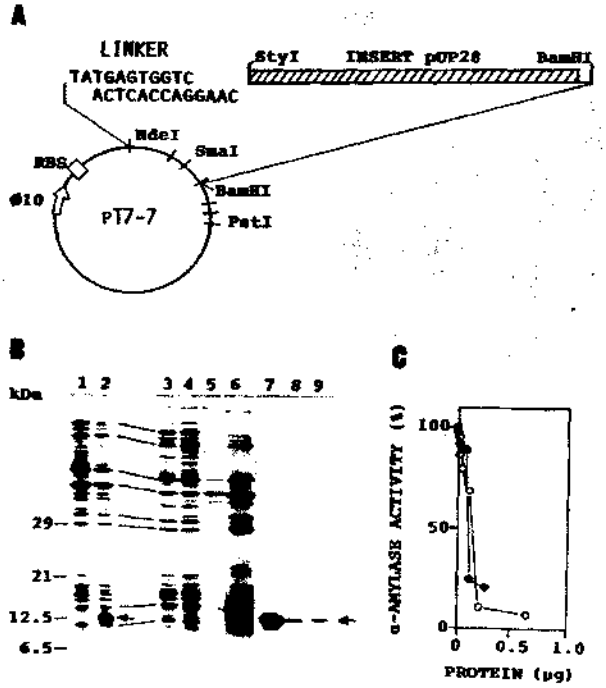

Fig. 1. Expression of WMAI-1 (syn. 0.28) in *E. coli*. A. Insertion of DNA encoding WMAI-1 in expression plasmid pT7-7, which contains a promoter for T7 RNA polymerase (φ 10) and a ribosome binding site (RBS). B. Expression and purification of WMAI-1. Indicated bacteria containing pT7-7 with (tracks 2, 4, 6, 7, 8) and without (tracks 1, 3, 5) the WMAI-1 insert were fractionated as described in Materials and methods: total extract from 125 μl of culture (1, 2); 25 μl of cytosolic fraction (3, 4); inclusion bodies equivalent to 125 μl of cytosolic fraction (5, 6); 15 μg of acetic acid extract (7); 5 μg of HPLC purified fraction (8); 5 μg of native WMAI-1 (9). Horizontal arrow points to WMAI-1 band. C. Inhibitory activity against the α-amylase of the insect *Tenebrio molitor* of the WMAI-1 produced in *E. coli* (●) and the native one isolated from wheat cv. Chinese Spring (○). Activity is represented as a percentage of the α-amylase activity obtained without inhibitors.

without insert and had the electrophoretic mobility of native WMAI-1 (Fig. 1B). This protein accumulated in inclusion bodies, from where it was purified by solubilization with 2 M guanidinium chloride, precipitation with 70% saturated $(NH_4)_2SO_4$, solubilization with 0.1 M acetic acid, and fractionation by RP-HPLC (Fig. 1B). The purified protein and native WMAI-1 from the same Chinese Spring wheat cultivar used to clone the cDNA had the same specific inhibitory activ-

ity (Fig. 1C). Correct processing of the terminal methionine was checked by amino acid N-terminal sequencing.

Mutants of WMAI-1

Three regions of WMAI-1 are rather conserved among the known monomeric and dimeric α-amylase inhibitors, while being quite divergent in the other members of the family [5, 9]: the N-terminal sequence before the first cysteine at position 7; the sequence between the 3rd and the 4th cysteines (positions 29–42); and a short sequence right after the 7th cysteine (position 56). The C-terminal half of the molecule is much variable among the different inhibitors [5]. A total of 13 mutants were obtained at 6 different sites (A through F in Fig. 2). Sites A–E were in the conserved regions, while site F was in a variable one. The mutant genes were sequenced and the alterations found with respect to the wild type are listed in Fig. 2A. The deduced amino acid sequences of the mutants are presented in Fig. 2B: mutant A1 consisted of a substitution of the terminal serine by a 6 amino acid sequence (ARIRAR) derived from the polylinker in pT7 7; mutants at sites C–E are all insertions; and mutants at site F involve the substitution of two residues by longer variant sequences. The positions of the mutation sites with respect to the predicted secondary structure of the wild type are represented in Fig. 2C.

Mutants were expressed in *E. coli* and purified to homogeneity following the same procedure as for the wild type. Their electrophoretic mobilities in SDS-PAGE are consistent with the changes introduced (Fig. 3).

Inhibitory activities of the mutants

All mutants were tested against the α-amylase of *Tenebrio molitor* with 40 min of preincubation (Fig. 4). Mutants B2, E1, E2, and E3 were completely inactive under these experimental conditions, while all others retained high levels of ac-

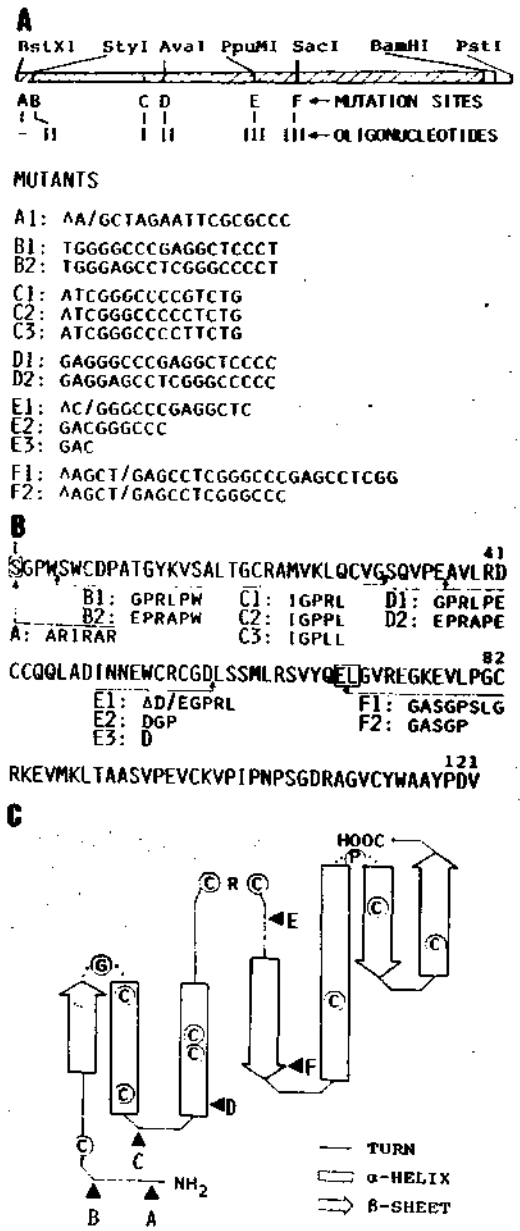

Fig. 2. Site-directed mutagenesis of WMAI-1. A. Mutation sites and mutant sequences determined by DNA sequencing. Mutation strategies and synthetic oligonucleotides used are described in Materials and methods. B. Deduced amino acid sequences of the mutants. Substitutions are boxed and insertions are indicated by an arrow. C. Mutation sites indicated in the predicted secondary structure of WMAI-1 ([6] and Microgenie computer program Beckman).

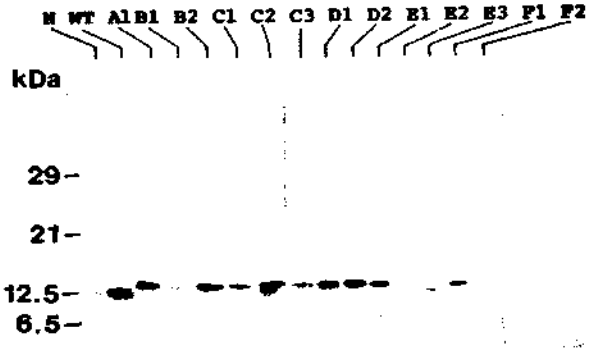

Fig. 3. Analysis of purified proteins by SDS-PAGE. N, native WMAI-1 from wheat cv. Chinese Spring; WT, wild-type WMAI-1 produced in *E. coli*; A1–F2, purified proteins from mutants. From mutants C1–3 the last two purification steps were ommitted. Gels were stained with Coomassie blue as indicated in Materials and methods.

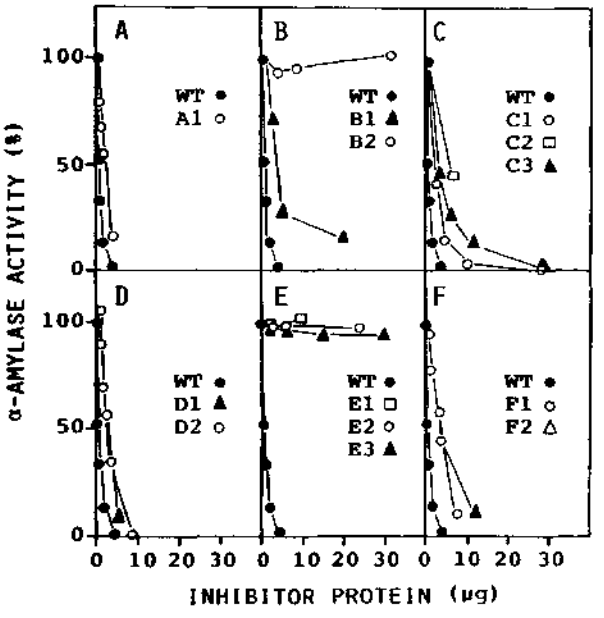

Fig. 4. Inhibitory activity of WMAI-1 mutants against the α-amylase from the insect *Tenebrio molitor*. Activity is represented as a percentage of the α-amylase activity in the absence of inhibitor. The wild-type (WT) inhibitor produced in *E. coli* was used for comparison. Enzyme and inhibitor were preincubated for 40 min before substrate was added.

tivity, although always below that of the wild type. As is the case with the native inhibitor from wheat, the wild type synthesized in *E. coli* is fully active

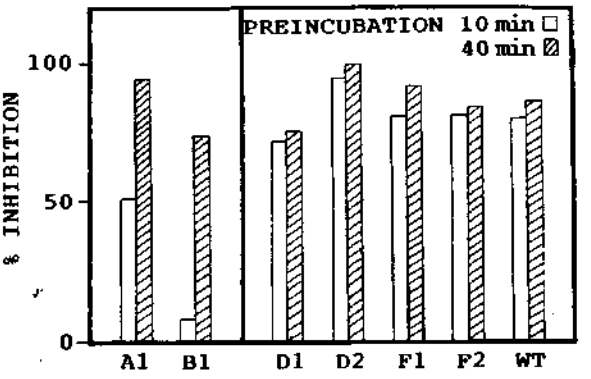

Fig. 5. Comparison of inhibitory activity at 10 min and at 40 min pre-incubation. Amounts of inhibitor used were the following : WT, 2 μg; A1, 4.5 μg; B1, 6 μg; D1, 5 μg; D2, 10 μg; F1, 7 μg; F2, 7.5 μg. Pre-incubation was at 25 °C.

after 10 min preincubation. In this respect, all mutants behaved as the wild type, with the exception of mutants A1 and B1, which required at least 40 min of preincubation to reach maximum activity (Fig. 5). None of the inhibitors was active against trypsin or chymotrypsin.

Discussion

The present evidence that WMAI-1 (syn. 0.28) synthesized in *E. coli* has the same electrophoretic mobility as the native one isolated from the endosperm of the same wheat cultivar used for the cDNA cloning, as well as the same specific inhibitory activity is in line with its failure to react with glycoprotein reagents when bound to filters (G. Salcedo, R. Sanchez-Monge and L. Gomez, unpublished) and directly contradicts previous claims that the inhibitor was glycosylated [15, 18] and that the sugar moiety played a key rol in the inhibition mechanism [19].

The mutants obtained allow the identification of two regions of the molecule that are critical for the inhibition mechanisms: the N-terminal sequence before the 1st cysteine (sites A and B), and site E, which is right after a CRC motif (positions 54–56) that is conserved throughout the family. The roles of the two regions seem to be

different, since the effect of mutations in the first region (A1, B1) affect the kinetics of formation of the enzyme-inhibitor complex and tend to have a moderate effect on inhibitory activity, whereas all the mutations at the E site, even a single amino acid insertion (E3), render the inhibitor completely inactive.

The substitution of the conserved N-terminal serine by a highly basic extension of six amino acids (A) had a more moderate effect on optimum preincubation time and on inhibitory activity than the insertions in mutants B1 and B2, which do not affect the conserved SGPW terminal motif. These two mutants, which only differed in one conservative (L/A) and one non-conservative (G/E) substitution, markedly diverged in their inhibitory properties. It seems that the G→E difference makes the N-terminal less flexible, which would explain the difference in activity between the two mutants. The times required by different plant proteinaceous inhibitors of α-amylases to react with α-amylase and to reach maximum inhibition range widely from 5 min to over 3 h and have been considered too long for a diffusion-controlled reaction between two proteins [18]. For this reason, an inhibitor-induced conformational change has been suggested as the rate-controlling step in the complexation (see [18]). In this context, the behaviour of mutants A1, B1, and B2 would indicate that the N-terminal of the inhibitor plays a role in the postulated conformational change and is less critical to the stability of the enzyme-inhibitor complex, for which the region around site E would be rather stringently critical.

The loop between the 3rd and 4th cysteines, which is quite conserved among the monomeric and dimeric α-amylase inhibitors, is also where the reactive site (GPRL) of those members of the family that inhibit trypsin is present. Mutations at two sites (C and D) within this loop had only a moderate effect on inhibitory activity. The introduction of the GPRL motif at these or at other sites did not lead to trypsin inhibition, indicating that other structural differences between members of the same family must be critical for this inhibitory activity.

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References

1. Barber D, Sanchez-Monge R, Gomez L, Carpizo J, Armentia A, Lopez-Otin C, Juan F, Salcedo G: A barley flour inhibitor of insect α-amylase is a major allergen associated with baker's asthma disease. FEBS Lett 248: 119–122 (1989).
2. Boisen D: Characterization of the endosperm trypsin inhibitor in barley. Phytochemistry 15: 641 (1976).
3. Erlanger BF, Kokowsky N, Cohen W: The preparation and properties of two new chromogenic substrates of trypsin. Arch Biochem Biophys 95: 271–278 (1961).
4. García-Olmedo F, Salcedo G, Sanchez-Monge R, Gomez L, Royo J, Carbonero P: Plant proteinaceous inhibitors of proteinases and α-amylases. Oxford Surv Plant Mol Cell Biol 4: 275–334 (1987).
5. García-Olmedo F, Salcedo G, Sanchez-Monge R, Hernandez-Lucas C, Carmona MJ, Lopez-Fando JJ, Fernandez JA, Gomez L, Royo J, García-Maroto F, Castagnaro A, Carbonero P: Alpha-amylase/trypsin inhibitors and thionins. Possible defense proteins from barley. In: Shewry PR (ed) Barley: Genetics, Molecular Biology and Biotechnology. CAB International (in press) (1991).
6. Garnier J, Osguthorpe DJ, Robson B: Analysis of the accuracy and implication of simple methods for predicting secondary structure of globular proteins. J Mol Biol 120: 97–120 (1978).
7. Gomez L, Sanchez-Monge R, García-Olmedo F, Salcedo G: Wheat tetrameric inhibitors of insect α-amylases: alloploid heterosis at the molecular level. Proc Natl Acad Sci USA 86: 3242–3246 (1989).
8. Gomez L, Martin E, Hernandez D, Sanchez-Monge R, Barber D, del Pozo V, Andres B, Armentia A, Lahoz C, Salcedo G, Palomino P: Members of the α-amylase inhibitors family from wheat endosperm are major allergens associated with baker's asthma. FEBS Lett 261: 85–88 (1990).
9. Gomez L, Sanchez-Monge R, Lopez-Otin C, Salcedo G: Wheat inhibitors of heterologous α-amylases: character-

ization of major components from the monomeric class. Plant Physiol 96: 768–774 (1991).
10. Gutierrez C, Sanchez-Monge R, Gomez L, Ruiz-Tapiador M, Castañera P, Salcedo G: α-Amylase activities of agricultural insect pests are specifically affected by different inhibitor preparations from wheat and barley endosperm. Plant Sci 72: 37–44 (1990).
11. Jonassen I, Svendsen I: Identification of the reactive sites in two homologous proteinase inhibitors isolated from barley. Carlsberg Res Commun 47: 199–203 (1982).
12. Kashlan N, Richardson M: The complete amino acid sequence of a major wheat protein inhibitor of α-amylase. Phytochemistry 20: 1781–1784 (1981).
13. Laemli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685 (1970).
14. Lazaro A, Sanchez-Monge R, Salcedo G, Paz-Ares J, Carbonero P, Garcia-Olmedo F: A dimeric inhibitor of insect α-amylase from barley. Cloning of the cDNA and identification of the protein. Eur J Biochem 172: 129–134 (1988).
15. Petrucci T, Sannia G, Parlamenti R, Silano V: Structural studies of wheat monomeric and dimeric protein inhibitors of alpha-amylase. Biochem J 173: 229–235 (1978).
16. Sanchez- Monge R, Gomez L, Garcia-Olmedo F, Salcedo G: A tetrameric inhibitor of insect α-amylase from barley. FEBS Lett 207: 105–109 (1986).
17. Sanger F, Nicklen S, Coulson AR: DNA sequencing with chain terminating inhibitors. Proc Natl Acad Sci USA 74: 5463–5467 (1977).
18. Silano V: Alpha-amylase inhibitors. In: Kruger JE, Lineback D, Stauffer CE (eds) Enzymes and their Role in Cereal Technology, pp. 141–199. AACC, St Paul, MN (1987).
19. Silano V, Pooerio E, Buonocore V: A model for the interaction of wheat monomeric and dimeric protein inhibitors with alpha-amylase. Mol Cell Biochem 18: 87–91 (1977).
20. Smith PK, Krohn RI, Hermanson ET, Maelia AK, Gastner FH, Provenzano MD, Fujimoto EK, Goeke NW, Olson BJ, Kenk DC: Measurement of protein using bicinchonimic acid. Anal Biochem 150: 76–85 (1985).
21. Tabor S, Ricardson CC: A bacteriophage T7 RNA polymerase promoter system for controlled exclusive expression of specific genes. Proc Natl Acad Sci USA 82: 1074–1078 (1985).
22. Walsh BJ, Howden MEH: A method for the detection of IgE binding sequences of allergens based on a modification of epitope mapping. J Immunol Meth 121: 275–280 (1989).